

EFFECT OF GROWTH HORMONE  
ON POLYAMINE SYNTHESIS IN RAT LIVER

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EFFECT OF GROWTH HORMONE  
ON POLYAMINE SYNTHESIS IN RAT LIVER

A thesis submitted  
in accordance with  
the requirements  
for the degree of

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by



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## ABSTRACT

One of the initial events in the response to exogenous growth hormone in rats is a marked increase in the activity of hepatic ornithine decarboxylase, the rate limiting enzyme for polyamine biosynthesis. It is thought that polyamines could participate in the control of protein synthesis, both at the level of transcription and translation. Therefore, it would appear probable that their synthesis could be an important step in the protein anabolic response of the liver to growth hormone.

Ornithine decarboxylase activity is generally assayed by the release of  $^{14}\text{CO}_2$  from ornithine-1- $^{14}\text{C}$ , but this carbon may also be released by the sequential action of the mitochondrial enzymes ornithine transaminase,  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase, glutamate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase. Therefore, the assay of ornithine decarboxylase in crude homogenate requires inhibition of this pathway. In the present study, aminooxyacetate ( $10^{-5}\text{M}$ ) inhibited over 98% of ornithine transaminase activity and virtually eliminated decarboxylation of ornithine by mitochondria, without any effect on cytoplasmic ODC activity. That the cytoplasmic activity represented true ODC was shown by the stoichiometric production of  $\text{CO}_2$  and putrescine from ornithine.

Subcellular fractionation of rat liver by differential centrifugation, followed by assay of ornithine decarboxylase in presence of aminooxyacetate and of putrescine dependent S-adenosyl methionine decarboxylase, ornithine transaminase and of marker enzymes from each fraction, demonstrated that ornithine decarboxylase and putrescine dependent S-adenosyl methionine decarboxylase are exclusively located in the

cytosol, while ornithine transaminase is exclusively located in the mitochondrial fraction. The greatly increased ornithine decarboxylase activity observed after growth hormone administration was also found to be localized entirely in the cytoplasm. The specific activities of putrescine dependent S-adenosyl methionine decarboxylase and ornithine transaminase showed no change 4 hours after growth hormone administration; their sub-cellular location also remained unaffected.

Km values for hepatic ornithine decarboxylase using ornithine as a substrate showed no significant differences between control and growth hormone treated rats. Both displayed Km values of about 32  $\mu$ M. The dissociation constants for putrescine as activator of putrescine dependent S-adenosyl methionine decarboxylase were also unchanged 4 hours after growth hormone injection (270  $\mu$ M).

Ornithine decarboxylase showed a maximal specific activity in liver cytosol at weaning in control rats and then decreased rapidly to a low level of activity about one week later. At all ages tested, growth hormone injection four hours prior to sacrifice resulted in an elevation of ornithine decarboxylase activity. However, this response was most marked in young rats (about weaning age) and in older rats (greater than five weeks of age). For reasons as yet not evident, the response in three to five week old male rats was greatly attenuated.

A steady increase in activity of ornithine transaminase was observed from neonates to one month of age. This activity was maintained into adulthood and then began to decline steadily in the older rats. There was no change in ornithine transaminase activity four hours after growth hormone administration at any of the ages tested.

Putrescine concentration in rat liver showed a progressive decline with age. A single injection of growth hormone (2 mg/100 g body weight) four hours before sacrifice resulted in an approximate doubling of putrescine concentration. Spermidine concentrations also declined with age, but were unaffected by growth hormone. The concentration of spermine increased to a maximal level at five weeks of age and then steadily declined. Growth hormone was also without effect on the concentration of this polyamine four hours after injection.

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## LIST OF ABBREVIATIONS

$\alpha$ KG	Alpha ketoglutarate
$\alpha$ KGDH	Alpha ketoglutarate dehydrogenase (1.2.4.2)
AON	Aminooxyacetate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
DOC	Deoxycholate
$\epsilon$	Molar extinction coefficient
GDH	Glutamate dehydrogenase (1.4.1.1)
H	Homogenate fraction
HSS (S)	High speed supernatant fraction
L	Lysosomal fraction
LDH	Lactate dehydrogenase (1.1.1.27)
M	Mitochondrial fraction
N	Nuclear fraction
OCT	Ornithine carbamyl transferase (2.1.3.3)
ODC	Ornithine decarboxylase (4.1.1.17)
OT	Ornithine transaminase (ornithine keto acid transferase) (2.1.3.3)
P	Microsomal fraction
PCDH	$\Delta^1$ pyrroline-5-carboxylate dehydrogenase (1.2.1.a)
SAM	S-adenosyl-methionine
SDC	Putrescine dependent S-adenosyl methionine decarboxylase
TRIS	Tris (hydroxymethyl) amino methane
GH	Growth hormone
RNA	Ribonucleic acid
tRNA	Transfer ribonucleic acid
rRNA	Ribosomal ribonucleic acid

## INTRODUCTION

### A. Effect of Growth Hormone on Liver

It is well known that a single injection of GH causes increased RNA and protein synthesis in rat liver within several hours ( 1 ). However, the mechanism of this anabolic effect remains to be elucidated.

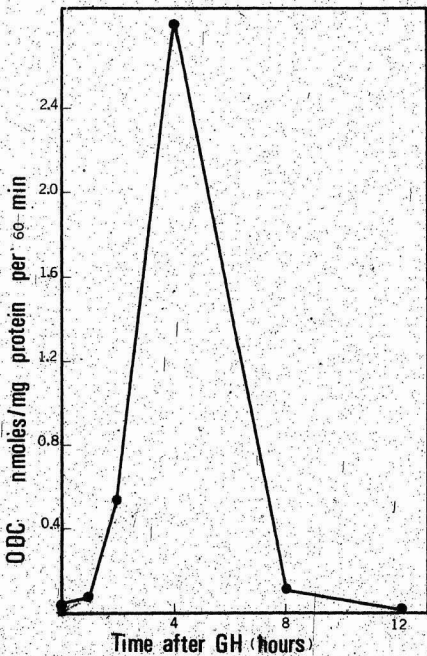
It has been proposed that the large polypeptide hormone interacts with a specific receptor on the plasma membrane of the liver cell and that this interaction is then translated into the biological response ( 2 ).

It is possible that hydrolytic cleavage of the hormone after binding allows "active moieties" of GH to penetrate the membrane and act directly on cellular processes ( 3,4 ). A more popular argument, analogous to the second messenger hypothesis of Sutherland, is that a hormone stimulates adenyl cyclase which then produces cAMP which acts as a second messenger, carrying out the physiological function of the hormone ( 5 ). Since cAMP appears to inhibit proliferation in some tissues ( 6 ), this is not a likely candidate for the growth hormone second messenger. However, recent evidence has implicated cGMP in the control of growth processes in a number of tissues ( 6a,6b ) and thus it is possible that it could be involved in the GH response. Alternatively, there could be another as yet unrecognized second messenger.

Raina and Holta ( 7 ) have demonstrated marked increases in the specific activities of hepatic ornithine decarboxylase (ODC) (Figure 1) and RNA polymerase 4 hours after GH injection to rats.

Figure 1

ODC activity as a function of time after growth hormone injection  
(redrawn according to Raina and Holttä, 7).



Ornithine decarboxylase is the first enzyme in the pathway of polyamine biosynthesis and is thought to be rate limiting (18). The increase in RNA polymerase activity appears to be less striking than the ODC elevation but nevertheless it is still considered quite significant. In this series of experiments, Jänne (1,8) observed increases in radioactive precursor incorporation into RNA and protein in rat liver 6 hours after GH injection. These experiments and the work of other investigators (9,10,11) demonstrate that one of the earliest responses of liver to GH is a rapid increase in the rate of synthesis of polyamines. Similar results were also observed when rat liver is perfused with a solution containing GH (7,12).

#### B. Synthesis of Polyamines

There are three main polyamines which occur in almost all eucaryotic cells. They are putrescine,  $\text{NH}_2(\text{CH}_2)_4\text{NH}_2$ , which is actually a diamine, spermidine,  $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$  and spermine,  $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$ . At physiological pH, these amines occur as polycations.

Taba et al. (13) showed that in *E. coli* and certain other microorganisms, putrescine could be derived from arginine and/or ornithine by decarboxylation of these amino acids. The propylamino group of spermidine is derived from S-adenosyl methionine (SAM). Polyamines in yeast and animal tissues (14-16) appear to be derived directly from ornithine only. It should be noted that the properties of the various enzymes involved in the biosynthesis of polyamines and their modes of regulation are quite different in eucaryotes from

Through the rest of this discussion putrescine will be classified as one of the three main polyamines.



those in procaryotes.

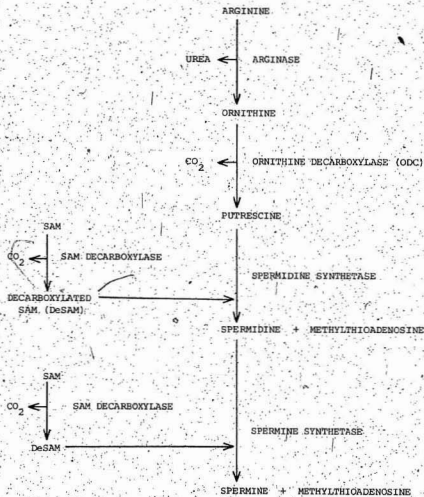
Figure 2 shows the eucaryotic pathway for the production of all three major polyamines ( 7 ). Arginine is converted to ornithine by arginase. Ornithine is then decarboxylated by ornithine decarboxylase ( 17 ) to produce the polyamine, putrescine. This enzyme is considered to be the rate limiting enzyme in the polyamine biosynthetic pathway ( 18 ). Experiments using inhibitors of protein synthesis have shown the apparent half-life ( $t_{1/2}$ ) of ODC in unstimulated rat liver to be approximately 11 minutes ( 19 ). This is the shortest  $t_{1/2}$  of any enzyme studied to date.

Spermidine is produced by spermidine synthetase action on putrescine and decarboxylated S-adenosyl methionine (SAM). A similar reaction catalyzed by spermine synthetase, results in formation of spermine from spermidine and decarboxylated SAM. It was originally thought that SAM itself was the substrate for these enzymes and that it was decarboxylated as part of the synthetic reaction. However, it has recently been demonstrated that S-adenosyl methionine decarboxylase exists as an independent entity, at least in rat prostate ( 20 ) and brain ( 21 ). Hannonen et al. ( 22 ) postulated that this is also probably the case in rat liver. Putrescine appears to act as an essential activator of S-adenosyl methionine decarboxylase ( 23 ). Thus spermidine synthesis would be highly dependent on the product of ODC activity, since putrescine is not only a substrate but also an essential activator of the synthesis of the second substrate. This would support the postulate that ODC appears to be the rate limiting enzyme in the synthesis of polyamines.

Polyamines are thought to be degraded by specific oxidases ( 24 ).

Figure 2

Biosynthetic Pathway of Polyamines in Eucaryotes.



The only polyamine oxidases studied in animals to date appear to be found in extracellular fluids (plasma, semen), where, it is thought, the resultant aldehydes play a bacteriocidal role ( 25 ). At present, very little is known about this family of enzymes or their regulation in animal cells.

C. Factors Which Regulate Ornithine Decarboxylase Activity and Polyamine Concentrations

According to Siimes ( 26 ) polyamines are found in highest concentrations in the most active protein synthesizing tissues of human (Table I). For example the pancreas, which produces insulin, glucagon and digestive enzymes, has relatively high concentrations of spermidine and spermine when compared to a tissue with a relatively low rate of protein synthesis, such as skeletal muscle. Polyamine (especially spermidine) concentrations are also very high in rapidly growing tissues, such as certain tumors ( 27 ). Neonatal animals have been shown to have elevated concentrations of polyamines, especially spermidine, which decrease as the animals reach maturity ( 28 ). Surgical ( 29 ) or chemical ( 30 ) damage of viable liver tissue results in rapid synthesis of new tissue to replace that which has been destroyed. Such regeneration of liver tissue is preceded by a rapid increase in ODC and by polyamine accumulation ( 29, 30 ). Growth promoting hormones such as hypophysial growth hormone ( 7 ) and insulin ( 31 ) also produce elevated concentrations of polyamines in various tissues.

In general, factors which stimulate growth in liver cells result in a rapid (within 2 to 4 hours) increase in both ODC and RNA polymerase

TABLE I

CONCENTRATION OF POLYAMINES IN SELECTED  
TISSUES OF HUMAN (ADAPTED FROM SIIMES (26) )

Tissue	Spermidine	Spermine
	(nmoles/mg protein)	
adrenals	0.68	5.44
brain	9.67	2.86
heart	0.11	2.05
skeletal muscle	0.10	1.40
kidney	0.63	3.56
liver	1.01	5.17
pancreas	11.40	21.16
spleen	1.28	5.45

activities and in accumulation of polyamines and RNA (10,32).

The mechanism(s) of ODC increase is still not understood. Byus and Russell (5), using rats exposed to cold and administered with aminophylline have claimed to have observed rapid increases in cyclic adenosine monophosphate (cAMP) in the adrenal medulla and adrenal cortex; followed by dramatic increases in ODC activity. They proposed a mechanism for the mediation of ODC activity by cAMP involving the activation of protein kinase. A rapid transient rise in cAMP concentrations followed by a peak increase in ODC activity, about 5 hours after cAMP has returned to normal, does not appear to be solid evidence for a correlation between cAMP production and ODC activity. Others have reported injections of dibutyryl cAMP resulting in significant increases in ODC activity in the adrenal glands (33) and in the liver (34,35). Injections of large amounts of such compounds as dibutyryl cAMP into animals do not necessarily indicate activation of ODC by cyclic nucleotides. Release of a number of hormones, most notably the adrenal steroids, is known to be mediated by cAMP. However, these investigators (33-35) did not measure plasma concentrations of any hormones after cAMP injection.

Several reports (5,19,36) have indicated a smaller increase in ODC activity when RNA and protein synthesis inhibitors, such as actinomycin D, puromycin and cycloheximide, are added before or during administration of stimuli known to increase ODC activity. They take this as evidence for de novo synthesis of ODC. This decrease in activity, however, should not be unexpected since ODC activity appears to be regulated in concert with RNA and protein synthesis, and thus inhibition of these processes might also be expected to result in inactivation of ODC.

A recent study by Mitchell and Sedory (18) indicates that this is precisely what occurs after cycloheximide treatment in Physarum polycephalum. McIlhinney and Hogan (37) have reported that turnover of ODC is decreased by growth stimuli in hepatoma cells. This would indicate that the increased activity of ODC is achieved by decreased degradation of the enzyme, rather than by increased synthesis. However, all  $t_{1/2}$  of ODC have been calculated by measuring loss of enzyme activity after inhibition of protein synthesis with cycloheximide. Therefore, this data should be held suspect until more convincing evidence can be obtained by measurement of enzyme protein.

#### D. Proposed Role(s) of Polyamines

Although a variety of effects of polyamines have been observed on protein and nucleic acid synthesis and on stabilization of nucleic acids in both procaryotes and eucaryotes, the true physiological role(s) of polyamines is still far from clear.

It is known that the positively charged polyamines bind tightly to nucleic acids and can produce precipitates if they are sufficiently concentrated (38). It appears there are non-covalent linkages between the basic groups of polyamines and the acidic phosphate groups of nucleic acids. The polyamines could align themselves in the helical loops of DNA (or looped RNA), binding phosphate groups on either side by electrostatic and hydrogen bonds with their amine and imine groups. This causes a neutralization of the negatively charged groups, which usually repel each other, thus increasing the strength of the secondary structure of DNA and looped RNA. Such a structural reinforcement has been shown to protect nucleic acids against thermal denaturation and enzyme degradation (39,40). This

stabilization could lead to longer half lives for both DNA and RNA.

Perhaps the most striking similarity between polyamine and nucleic acid regulation is the strong parallel observed between the concentration of spermidine and RNA, especially rRNA ( 41 ). For example, it has been reported that spermidine concentration decreases with age in rat liver, as does RNA content ( 28 ). In regenerating rat liver, increased spermidine concentration is closely correlated with increased RNA concentration ( 42 ). Many other investigators have also observed a strong correlation between rRNA synthesis in both procaryotes and eucaryotes (43,44,45). It has been postulated ( 41 ) that polyamines could stabilize RNA, thus preventing its degradation, and therefore promoting its movement from the nucleus to the cytoplasm. It has also been suggested that polyamines could increase the rate of ribosome assembly ( 46 ). Either postulate would lead to a more efficient use of the RNA synthesized in the nucleus and therefore apparently more efficient transcription. Whether spermidine synthesis precedes RNA (especially rRNA) synthesis is still not clear. Most research on growth stimulation appears to indicate a simultaneous increase in the synthesis of spermidine and RNA (8,47), which would indicate co-ordinate control of these processes.

Polyamines have also been demonstrated to increase the synthesis of polypeptides at the translational level of protein synthesis. Spermidine and spermine appear to have an effect on the formation of aminoacyl transfer RNA ( 48 ) in E. coli. Cohen ( 49 ) has postulated that polyamines may stabilize transfer RNA (tRNA) in a favorable conformation. He observed that the biological activity of tRNA appeared to be a function of tRNA polyamine or cation (eg. magnesium)



content in E. coli. Takeda (50;51) observed that polyamines affected the binding of tRNA to ribosomes. He also postulated that polyamines affect the binding of messenger RNA to ribosomes, although he did not present evidence to support this idea.

There are many reports of polyamines binding to ribosomes (52,53), presumably as an integral part of the ribosome. Increased polyamine concentrations promoted aggregation of ribosomal subunits (54-57) and aided in the attachment of free ribosomes to cellular membranes (58). In all of these studies a critical minimum level of magnesium ions was necessary, but magnesium could not completely replace the polyamines.

# PROBLEMS OF INVESTIGATION

Since a dramatic increase in the specific activity of ornithine decarboxylase is one of the earliest effects of growth hormone action on rat liver, it is very important to know the sub-cellular location of this enzyme. Such knowledge could give some clues about the mode of action of the hormone on hepatic cells and possibly also about the function(s) of polyamines. It may be that growth hormone controls ODC via a second messenger, such as a cyclic nucleotide or that the enzyme is plasma membrane bound, whereby GH could directly affect its activity. It is also possible that there are isoenzymes of ODC in different locations of the cell which are hormonally controlled, as has been shown for tyrosine aminotransferase (59). Therefore, a sub-cellular localization of the enzyme in both control and GH stimulated rats was performed.

In view of the current lack of knowledge of the details of spermidine and spermine synthesis, we also investigated SDC, both with respect to sub-cellular localization and activity after GH injection, since SDC is involved in the synthesis of both of these polyamines and since the rate of spermidine synthesis has been shown to increase after GH injection (47). It has been suggested that it regulated the hepatic ornithine concentration and, this in turn, may control ODC (88). We, therefore, also examined this enzyme.

Some work previously indicated that very young rats were not responsive to GH and that adults lose responsiveness to certain effects of GH, especially in muscle (60) and cartilage (61). Since it has been reported that male rats will continue to grow if given exogenous GH (62), the growth related response in the liver might be expected

to continue. It was, therefore, decided to investigate the effects of GH on hepatic ODC activation in rats of different ages. In each of these rats, ornithine transaminase activity and polyamine concentrations were also monitored.

# MATERIALS AND METHODS

## Animals:

Male rats of the Sprague-Dawley strain were obtained from the animal unit, Faculty of Medicine, Memorial University of Newfoundland.

Growth hormone was dissolved in 0.9% saline (approx. pH 8-9) to give a concentration of 2 milligrams per millilitre. This solution was injected subcutaneously at a final dose of 2 milligrams growth hormone per 100 grams body weight. Control rats received a proportional subcutaneous injection of saline. Animals were sacrificed four hours after injection. To avoid diurnal variation in response, injections were always carried out between 9 and 10 A.M.

## Chemicals:

Growth hormone (NIH - GH - B16 Bovine) was a gift of the Endocrinology Study Section, NIAMD, Bethesda, Maryland.

L-ornithine ( $1-^{14}\text{C}$ ) (specific activity 7.7 mCi/mole) and NCS were purchased from Amersham-Searle Corporation, Oakville, Ontario.

S-adenosyl-L-methionine (carboxyl- $^{14}\text{C}$ ) (specific activity 7.7 mCi/mole),  $\alpha$ -Ketoglutaric acid ( $1-^{14}\text{C}$ ) (specific activity 1.4 mCi/mM), and Glutamic acid ( $1-^{14}\text{C}$ ) (specific activity 206 mCi/mole) and omnifluor were obtained from New England Nuclear, Dorval, Quebec.

Diphenylamine (Analar grade) was purchased from BDH Chemicals Ltd., Poole, England.

Phenolphthalein mono- $\beta$ -glucuronic acid (sodium salt), o-amino-benzaldehyde, and crystalline pyridoxal phosphate were purchased from Sigma Chemical Company, St. Louis, Mo.

All other chemicals, unless otherwise specified, were of the highest analytical grade and were obtained from either Sigma Chemical Company, St. Louis, Mo. or Fisher Scientific, Montreal, Quebec.

Centre wells and rubber stoppers used in radioactive carbon dioxide trapping were purchased from Kontes Glass Company, Vineland, New Jersey.

#### Tissue Homogenization and Isolation of Sub-cellular Fractions

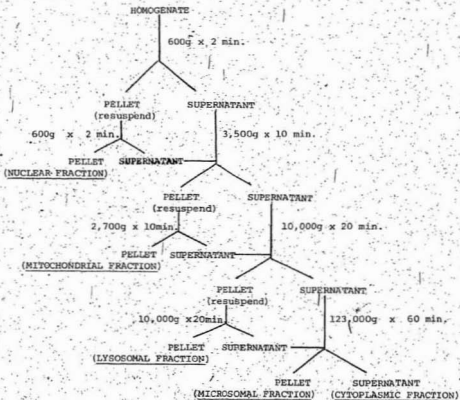
Rats were sacrificed by cervical dislocation. Livers were quickly removed and placed in approximately ten volumes of isolation medium (0.33M sucrose, 0.005  $MgCl_2$ , 0.01M Tris, pH 7.3). The liver was chopped with scissors, then homogenized in a smooth glass Potter-Elvehjem homogenizer at approximately 500 revolutions per minute by 5 to 6 strokes of a motor driven loose-fitting Teflon pestle (clearance 0.3 mm). After filtration through two layers of cheese-cloth, the homogenate was fractionated by differential centrifugation into a nuclear fraction (N), a mitochondrial fraction (M), a lysosomal fraction (L), a microsomal fraction (P) and a soluble fraction (S). The scheme proposed by DeDuve et al. (63), as modified by Sedgwick and Hubscher (64) was closely followed. This procedure is outlined in the flow sheet (Figure 3). All operations were carried out at 4°C.

#### Preparation of Mitochondria and High Speed Supernatant

When preparing only mitochondria, the method of Chappell and Hansford (65) was employed. The livers were homogenized in 2-5 volumes of medium (0.25M sucrose, 0.001M EGTA, 0.003M Tris, pH 7.4).

Figure -3

Flow chart for the sub-cellular fractionation of rat liver cells.



The supernatant obtained after the mitochondria were pelleted was further centrifuged to obtain the high speed supernatant. These procedures are outlined in Figure 4.

#### Markers for Subcellular Fractions

Fractions obtained by differential centrifugation were identified by measurement of the specific activities of the following "markers":

<u>Fraction</u>	<u>Marker</u>
nuclei	DNA
mitochondria	succinate cytochrome C reductase
lysosomes	$\beta$ -glucuronidase
microsomes	NADPH cytochrome C reductase
supernatant	lactate dehydrogenase

All enzyme assays were demonstrated to be linear with time and protein concentration under the conditions employed.

DNA: extracted into hot perchloric acid, according to the method of Schneider (66). DNA concentrations were determined by the diphenylamine method of Burton (67).

Succinate cytochrome C reductase: assayed according to the method of Sottocase et al. (68). The reduction of cytochrome C by succinate is catalyzed by an early portion of the respiratory chain. The addition of cyanide prevents the reoxidation of cytochrome C by the latter portion of the electron transport chain. The reaction was initiated by the addition of succinate, and optical density was monitored at 550 nm. Enzyme rates were calculated using the (reduction - oxidation) extinction coefficient for cytochrome C  $\epsilon = 18.5 \text{ mM}^{-1} \text{ cm}^{-1}$ , 1 cm light path.

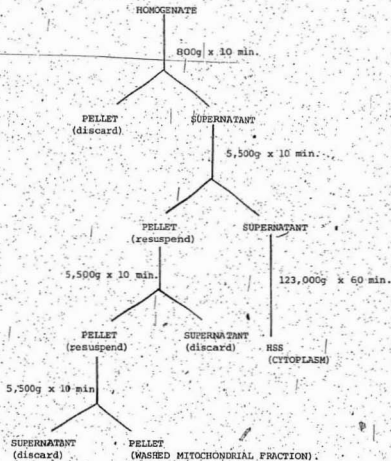
$\beta$ -glucuronidase: assayed according to Glanetto and DeDuve (69).





Figure 1.

Flow chart for the preparation of mitochondria and HSS from  
rat liver.



At acid pH, lysosomal  $\beta$ -glucuronidase hydrolyses phenolphthalein  $\beta$ -glucuronide to  $\beta$ -glucuronic acid and phenolphthalein. Addition of alkali terminates the reaction and produces the bright red colour typical of phenolphthalein in alkaline solution. The optical density was read at 560 nm and a standard curve was used to convert absorbance to moles of phenolphthalein released. Enzyme rates could then be calculated.

NADPH cytochrome C reductase: Assayed according to Sottocase et al. (68). The principle of this assay is exactly similar to that of succinate cytochrome C reductase, except that reduction of cytochrome is carried out by the microsomal electron transfer chain. The reaction was initiated by the addition of NADPH. The optical density was monitored at 550 nm. Enzyme rates were calculated using the (reduction - oxidation) extinction coefficient for cytochrome C ( $\epsilon = 18.5 \text{ mM}^{-1}$ , 1 cm light path).

Lactate dehydrogenase: Assayed according to Morrison et al. (70). The enzyme was assayed in the direction of NADH oxidation. The reaction was started by the addition of sodium pyruvate and optical density was recorded by 340 nm. Enzyme rates were calculated using the extinction coefficient for NADH ( $\epsilon = 6.22 \text{ mM}^{-1}$ , 1 cm light path).

#### Presentation of Data on Subcellular Localization

The results obtained from the fractionation studies were presented in the manner proposed by DeDuve et al. (63). In a DeDuve plot, the relative protein content of fractions (cumulatively from left to right on the abscissa) is plotted against relative specific activity (ordinate). The mean relative specific activity of fractions

is defined as the percentage of total activity for each fraction divided by the percentage of total protein for each fraction. The total activity of all enzymes is usually taken to be that activity found in the homogenate fraction. The total protein content is computed by addition of the total protein in each fraction. Because of the unusually high percentage recovery (total activity of all fractions/homogenate activity) of ornithine decarboxylase (ODC), its total activity was computed in a similar manner to that described for protein.

If a particular fraction shows a relative specific activity greater than 1.0, this would indicate that the enzyme in question has enriched activity in that particular fraction. By comparing the subcellular distribution patterns (DeDuve histograms) of each enzyme whose localization is uncertain with those of the marker enzymes, one can determine in which subcellular fraction most of the activity of each enzyme is present. Further details of this procedure can be obtained in the paper by DeDuve (63).

#### Assay for Ornithine Decarboxylase (ODC)

ODC activity was measured by following the release of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -L-ornithine, using a modification of the method of Pegg et al. (71). Reaction mixtures contained 0.1  $\mu\text{moles}$  pyridoxal phosphate, approximately 4 mg protein, 7.5  $\mu\text{moles}$  Tris, pH 7.3, 0.15  $\mu\text{moles}$  EDTA, 0.1  $\mu\text{moles}$  L-ornithine ( $1\mu\text{Ci}/\mu\text{mol}$ ) in a final volume of 2.0 ml. During the experiments to localize ODC, aminoxyacetate was added to all fractions at a final concentration of  $10^{-5}\text{ M}$ . Mixtures were incubated at  $37^\circ\text{C}$  for 10 minutes before the

reaction was initiated by addition of isotope. The reactions were carried out in glass Erlenmeyer flasks equipped with tight fitting rubber stoppers; each flask carried a polypropylene centre well in which was placed 0.2 ml of NCS and a small piece of filter paper. After 60 minutes of incubation, the reaction was terminated by addition of 0.2 ml of 5 N  $\text{H}_2\text{SO}_4$ , injected through the rubber stopper. Shaking was then continued for 60 min to collect radioactive carbon dioxide (91). Centre wells were quickly placed in toluene containing 4 grams/litre  $\text{Bmifluor}$  and radioactivity was measured using a Beckman LS-233 liquid scintillation counter. Corrections for quenching were made using the channels ratio method. Mixtures containing acid at time zero or containing no enzyme were used as blanks.

#### Assay of Putrescine-dependent S-adenosyl-L-methionine-Decarboxylase (SDC)

Enzyme activity was determined by measuring the liberation of  $^{14}\text{CO}_2$  from S-adenosyl-L-methionine (carboxyl- $^{14}\text{C}$ ) (72). The same basic method of  $^{14}\text{CO}_2$  trapping as described previously was used. Incubation mixtures contained 0.1  $\mu\text{moles}$  pyridoxal phosphate, 0.4  $\mu\text{mole}$  S-adenosyl methionine (0.5  $\mu\text{Ci}/\mu\text{mole}$ ), 3 to 10  $\text{mg}$  protein, 5  $\mu\text{moles}$  putrescine, 200  $\mu\text{moles}$   $\text{NaH}_2\text{PO}_4$ , pH 7.0, in a final volume of 2ml. Assays were carried out at  $37^\circ\text{C}$  for 60 minutes. All values were corrected by subtracting the value obtained when no putrescine was added.

#### Assay of Ornithine Transaminase

Ornithine transaminase (ornithine - 2-oxo acid aminotransferase)

activity was assayed by measuring the formation of glutamic  $\gamma$ -semi-aldehyde by the procedure of Peraino and Pitot (73), as modified by Pegg and Williams-Ashman (74). The reaction mixture contained 0.125  $\mu$ moles  $\text{KH}_2\text{PO}_4$ , pH 7.6, 100  $\mu$ moles L-ornithine, 20  $\mu$ moles o-aminobenzaldehyde (dissolved in 50  $\mu$ l 40% ethanol) and approximately 2 to 3 mg protein, in a total volume of 1.0 ml. The assay mixture was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 2.0 ml of 10% (w/v) trichloroacetic acid. Protein was removed by centrifugation. Optical density of the supernatant was measured at 440 nm. The amount of  $\delta$ -pyrroline-5-carboxylic acid (the spontaneous cyclized form of glutamic  $\gamma$ -semialdehyde) present was calculated, using the molar extinction coefficient for the coloured complex produced with o-aminobenzaldehyde ( $\epsilon = 2710$ ) (75).

#### Protein Assay

Protein was determined by the Biuret method (76), following solubilization of membranous material with deoxycholate (77). This was necessary to prevent turbidity in the assay. Bovine serum albumin was used as standard.

#### Determination of Polyamine Concentrations

Concentrations of polyamines were determined by the use of the Beckman 121 amino acid analyzer. A column of spherical sulphonated polystyrene resin 8% divinylbenzene cross linked, size 5 - 12 microns (Beckman PA-35 resin) was used.

Polyamines were eluted from a 41 x 12 mm column of PA-35 resin at 55°C, using sodium citrate buffer (pH 5.42/2.35 N,  $\text{Na}^+$ ) at a flow rate of 90 ml/hr. The polyamines were eluted as follows:

putrescine, cadaverine, spermidine, and spermine at 22, 32, 42 and 95 minutes respectively, after placement of sample on column.

The results were compared to standard concentrations of 0.25  $\mu$ moles/ml of each of the polyamines prepared in a sodium citrate buffer (pH 2.2/0.2 N  $\text{Na}^+$ ).

All samples were deproteinized by addition of 50 mg of sulphosalicylic acid per millilitre of sample. The resulting mixture was centrifuged at 6,800 g for 20 minutes.

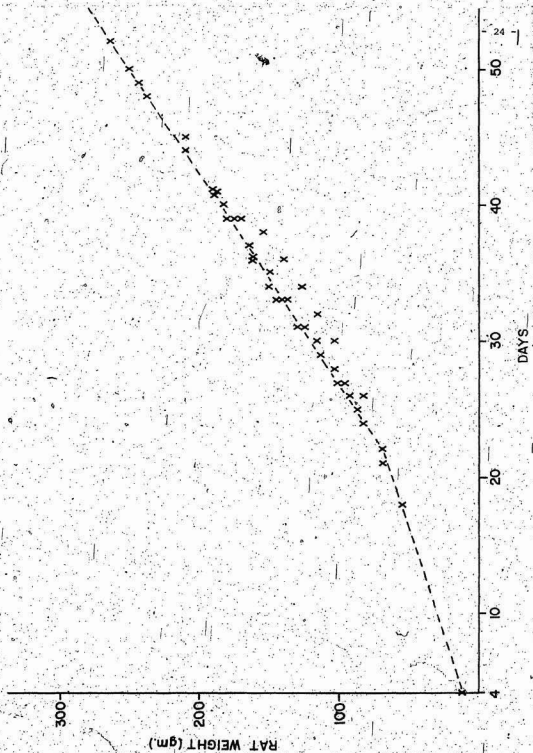
#### Growth Curve for Sprague-Dawley Rats

Rats were weighed immediately before injection each day. As these rats were obtained directly from the Memorial University of Newfoundland Medical School, exact ages were not available for them. An approximate growth curve has been constructed (Figure 5), but it is recognized that extrapolation from weight to age using this curve could give rise to an error of 10-15% due to individual variability of the animals. Therefore, all results are initially given by weight of rat (see especially Section D, Results and Discussion). However, it has been necessary to estimate ages of rats in the discussion of our results so that they can be compared to those of other investigators.

Figure 5

Growth curve of male Sprague-Dawley rats obtained from Animal Unit, Faculty of Medicine, Memorial University of Newfoundland.





## RESULTS AND DISCUSSION

A. Modifications of ODC Assay

Since ODC catalyzes the formation of one mole of  $\text{CO}_2$  and one mole of putrescine from one mole of ornithine, it should be possible to assay this enzyme by measuring appearance of either product. In practice, this assay is generally carried out by following production of  $^{14}\text{CO}_2$  from 1- $^{14}\text{C}$  ornithine, as this measurement is simple, reproducible and extremely sensitive (71). Formation of  $^{14}\text{C}$  putrescine from 5- $^{14}\text{C}$  ornithine can be assessed by applying the sample to an amino acid analyzer (see Methods section K), collecting the fractions off the column and then measuring radioactivity in each. This method requires far more material for accurate measurement but it is open to fewer artifacts.

Thus, it was desirable to carry out a preliminary assay in which both  $\text{CO}_2$  and putrescine formation were monitored to determine suitability of  $^{14}\text{CO}_2$  production as a measure of ODC activity.

A trial fractionation indicated that activity followed both the cytoplasmic marker, LDH, and the mitochondrial marker, succinate cytochrome C reductase, if the  $^{14}\text{CO}_2$  trapping method were used. In the HSS, it was found that there was a stoichiometric relationship between  $^{14}\text{CO}_2$  and  $^{14}\text{C}$  putrescine produced (Table 2). Therefore, in this fraction the  $^{14}\text{CO}_2$  method appeared to be a true measure of ODC activity. However, in the mitochondrial fraction, formation of putrescine equivalent to the  $\text{CO}_2$  released could not be shown (Table 2). In hepatic mitochondria ornithine could either be converted to citrulline by ornithine transcarbamylase (78) or to glutamic  $\gamma$  semialdehyde by the enzyme

TABLE 2

COMPARISON OF TWO ASSAYS FOR ORNITHINE DECARBOXYLASE  
IN RAT LIVER CYTOSOL AND MITOCHONDRIA

Fraction	Radioactive Product (nmole/60min)		Ratio CO <sub>2</sub> /Putrescine
	Putrescine	CO <sub>2</sub>	
Cytosol	0.47	0.46	1.0
Mitochondria	0.42	18.39	43.8

Figure 6

Oxidative Metabolism of Ornithine in Hepatic Mitochondria.

The Labelled 1-carbon of L-ornithine is denoted by the symbol (C).

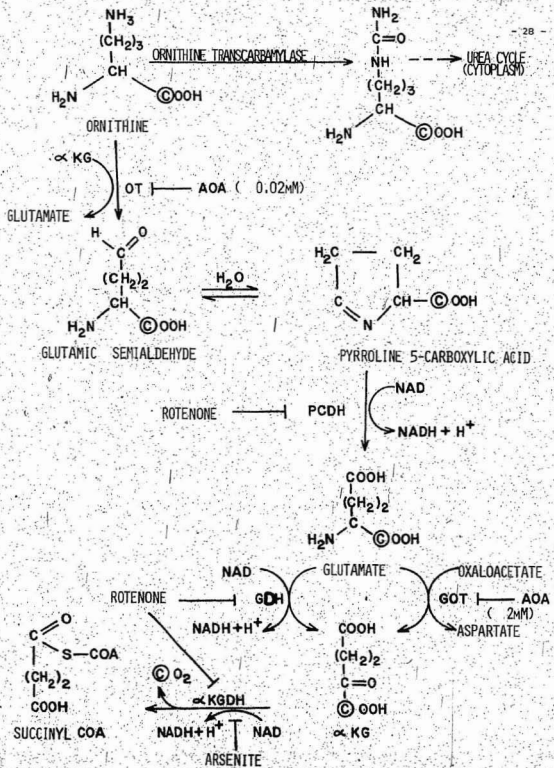
Sites of AOA, rotenone and arsenite inhibitor are also denoted

(—+)

ornithine transaminase (OT). In the case of ornithine transcarbamylase the product citrulline, which is an intermediate in the urea cycle, is transported out into the cytoplasm. Thus this pathway would offer no mode of release for  $^{14}\text{CO}_2$  from L- $^{14}\text{C}$ -ornithine in mitochondria. In the case of ornithine transaminase, the product glutamic  $\gamma$  semialdehyde can be metabolized to glutamate by the action of  $\delta^1$  pyrroline 5-carboxylic acid dehydrogenase (79). In hepatic mitochondria glutamate can be metabolized to  $\alpha$ -ketoglutarate ( $\alpha\text{KG}$ ) by both glutamate dehydrogenase and glutamate oxaloacetate transaminase. As shown in figure 5, the labelled carbon from the L-ornithine (L- $^{14}\text{C}$ ) becomes the 1-C of  $\alpha$ -ketoglutarate. This labelled carbon can be removed from the  $\alpha\text{KG}$  as  $^{14}\text{CO}_2$  by the action of the mitochondrial enzyme,  $\alpha\text{KG}$  dehydrogenase (67).

Therefore, before a sub-cellular localization of ODC could be carried out, it was necessary to determine if this alternate pathway was involved in mitochondrial  $^{14}\text{CO}_2$  release from L-ornithine (L- $^{14}\text{CO}_2$ ) under the conditions of our ornithine decarboxylase assay. If this were the case, it would be necessary to inhibit the OT to prevent artifacts in the ODC localization experiments.

In order to determine what percentage, if any, of the ornithine was metabolized via the glutamate- $\alpha$ -ketoglutarate pathway, a number of inhibitors were employed. The inhibitor rotenone was used to inhibit any nicotinamide adenine dinucleotide (NAD) dependent steps (80). Inspection of the pathway (see Figure 6) indicates three possible sites of rotenone inhibition. The mitochondrial NAD-requiring enzymes involved are a)  $\delta^1$  pyrroline 5-carboxylic-acid dehydrogenase, b) glutamate dehydrogenase and c)  $\alpha\text{KG}$  dehydrogenase. Arsenite was used



to inhibit  $\alpha$ -ketoglutarate dehydrogenase (81). Aminooxyacetate (AOA) was reported by Rognstad (82) to specifically inhibit GOT in lower concentration (0.2mM) than is required to inhibit most other transaminases. Thus AOA was used initially to inhibit GOT.

In these studies three different labelled substrates, thought to be intermediates in the pathway between ornithine and succinyl CoA were used. These were L-ornithine, L-glutamate and  $\alpha$ -ketoglutarate, all labelled on the same carbon (1- $^{14}\text{C}$ ). Mitochondrial  $^{14}\text{CO}_2$  evolution was assayed as described in Methods in ODC, using one of the three labelled substrates with each different inhibitor (Table 3).

Rotenone inhibited  $^{14}\text{CO}_2$  production by mitochondria by approximately 89-95% when ornithine or glutamate were used and by approximately 75% when  $\alpha$ -ketoglutarate was the labelled substrate. On the other hand, rotenone was without effect on cytoplasmic  $^{14}\text{CO}_2$  production. These results alone would seem to indicate that at least 90% of the labelled  $\text{CO}_2$  evolved from ornithine (1- $^{14}\text{C}$ ) was not removed by ornithine decarboxylase but rather by the proposed pathway via glutamate.

When arsenite was added to a mitochondrial assay mixture containing either radioactive  $\alpha$ KG or glutamate, an inhibition of  $^{14}\text{CO}_2$  release of approximately 90% was observed, but, if ornithine were used as the labelled substrate, an inhibition of approximately 50% was detected. There was no effect of arsenite on cytoplasmic ODC activity. These results would indicate that only about half of the  $\text{CO}_2$  removed from ornithine was derived from the action of  $\alpha$ KG DH and that the other half was removed by some other pathway. The additional action of rotenone in this case could be to decrease the supply of  $\alpha$ KG required by ornithine transaminase. The identity of

TABLE 3

EFFECTS OF INHIBITORS ON MITOCHONDRIAL  $^{14}\text{CO}_2$  EVOLUTION USING  
LABELLED ORNITHINE, GLUTAMATE AND  $\alpha$ -KETOGUTARATE AS SUBSTRATES

Substrate	Inhibitor		
	Rotenone (.01mM)	Arsenite (1mM)	ACA (2mM)
	% inhibition	% inhibition	% inhibition
L-ornithine ( $1\text{-}^{14}\text{C}$ )	89.0	45.0	99.4
L-Glutamate ( $1\text{-}^{14}\text{C}$ )	95.4	88.5	51.9
$\alpha$ -Ketoglutarate ( $1\text{-}^{14}\text{C}$ )	73.4	89.2	0-200



the other pathway is not evident at this time.

By use of the glutamate-oxaloacetate transaminase (GOT) inhibitor, AOA (2mM) it was shown that, when labelled glutamate is added approximately 50% of the  $^{14}\text{CO}_2$  is inhibited under our conditions. When ornithine was used as the substrate it was observed that concentrations between 0.2mM and 2mM AOA would almost completely inhibit any  $^{14}\text{CO}_2$  evolution (98-99.4% inhibition). On the other hand, a concentration of 0.2mM AOA only inhibited glutamate decarboxylation by approximately 27%.

These results would indicate that some enzyme involved in converting ornithine to glutamate is much more sensitive to AOA than is GOT itself. Since AOA is known to inhibit pyridoxal phosphate-requiring enzymes, especially transaminases, it appeared likely that the sensitive enzyme was ornithine transaminase. Therefore, the next series of experiments was designed to test the possibility that addition of AOA could inhibit the oxidation of ornithine by liver mitochondria and thus permit accurate measurement of ODC activity by measuring  $\text{CO}_2$  production.

AOA,  $\text{NH}_2\text{-O-CH}_2\text{-COOH}$ , which is known to be a general transaminase inhibitor, apparently forms an oximino bond between the aminoxy group of the inhibitor and the aldehyde carbon of the transaminase co-enzyme, pyridoxal phosphate. The AOA carboxyl group is thought to form an electrostatic bond with a basic lysine nitrogen on the transaminase (83).

Hepatic mitochondria and HSS of normal male rats (approximately 100g) were prepared and assayed for OT activity as described in the methods. Varying amounts of AOA to give final concentrations up to

0.1mM were added. Activity was calculated as a percentage of the activity in the absence of AOA. The same spectrum of AOA concentrations was used in the assays for the mitochondrial and HSS  $^{14}\text{CO}_2$  evolution using L-ornithine ( $1\text{-}^{14}\text{C}$ ) as substrate.

Figure 7 shows that at a final concentration of 0.01mM AOA, mitochondrial ornithine transaminase activity was depressed by 75% and was completely inhibited by 0.1mM AOA. Mitochondrial  $^{14}\text{CO}_2$  evolution was almost completely inhibited at 0.01mM AOA (99%) while the HSS  $^{14}\text{CO}_2$  evolution was unaffected at 0.01 mM.

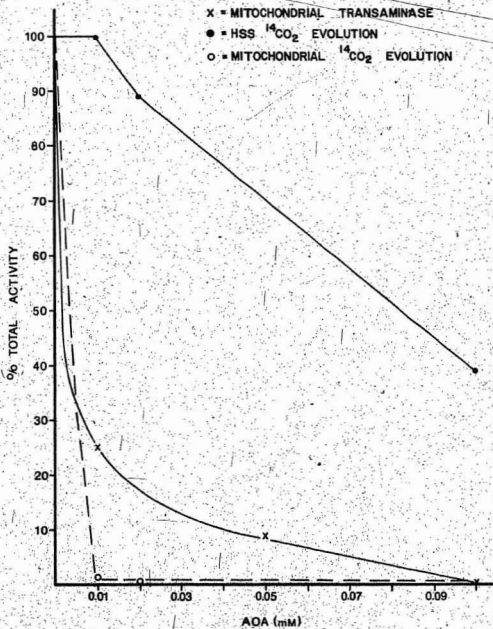
Therefore, it appeared that the use of 0.01mM AOA in all ODC assays would ensure against artifacts caused by the oxidative metabolism of ornithine via OT without seriously affecting the measurement of ODC activity.

It is interesting to note that at 0.01mM AOA, mitochondrial ornithine transaminase is inhibited by about 75% while the mitochondrial  $^{14}\text{CO}_2$  evolution was inhibited by 99%. This discrepancy could be due to one of several reasons. The AOA certainly inhibited  $^{14}\text{CO}_2$  evolution by blocking GOT besides ornithine transaminase (Table 3, column 3). The difference in assay conditions between OT and  $^{14}\text{CO}_2$  evolution assays might also be responsible for the difference in response of the two assays to AOA. The ornithine transaminase assay medium is isotonic while the  $^{14}\text{CO}_2$  evolution assay medium is hypotonic. The concentrations of substrates were also much higher in the OT assay than in the decarboxylation assay.

Figure 7

Enzyme activity as a function of AOA concentration.

- (● — ●) ornithine decarboxylation, fat liver HSS;
- (○ ---- ○) ornithine decarboxylation, rat liver mitochondria;
- (x — x) ornithine transaminase, rat liver mitochondria.



B. Sub-cellular Localization of Ornithine Decarboxylase, S-adenosyl Methionine Decarboxylase and Ornithine Transaminase in Livers of Control and Growth Hormone-Treated Rats.

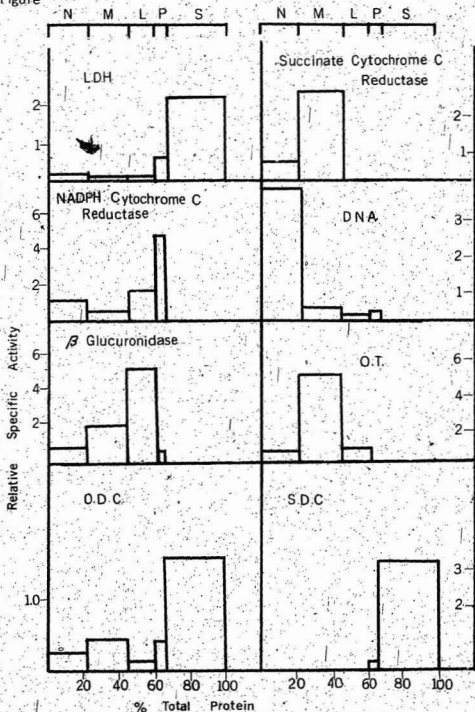
Sub-cellular fractionations and enzyme assays were carried out as described in the Methods. Marker enzyme activities and DNA content were measured in all fractions to compare their distribution with those of the enzymes under study (ODC, SDC and OT). The results obtained are presented in the manner proposed by DeDuve et al. (63) as described in the Methods section. The recoveries of enzymes, DNA and protein ranged from 80 to 110% with the exception of ODC, which gave a consistent recovery of approximately 160%. The reason for such a discrepancy remains unclear. It may be that there is an ODC inhibitory factor in the crude homogenate which is not present in the HSS. A deviation from linearity was sometimes observed with increasing volume of homogenate, which would lend credence to this interpretation. The distribution of marker enzymes was comparable to that found by other investigators (63) (84).

From figure 8, it can be seen that the sub-cellular distribution of ODC in liver from control rats was very similar to that of the cytosolic (HSS) marker enzyme, lactic dehydrogenase (LDH). This would indicate that ornithine decarboxylase is enriched in the HSS fraction (cytosol) of rat liver cells. SDC also showed the same distribution as LDH; again indicating a cytosolic location in the liver of control rats. Ornithine transaminase, on the other hand, showed a sub-cellular distribution similar to that of the mitochondrial marker enzyme, succinate cytochrome C reductase. Other workers (73) have previously reported

Figure 8

Typical distribution pattern of ODC, SDC, OT and marker enzymes, following sub-cellular fractionation of liver from control rat (rat weight: 92g). ordinate: mean relative specific activity of fractions (percentage of total activity/percentage of total protein); abscissa: relative protein content of fractions (cumulatively from left to right): N, nuclear fraction; M, mitochondrial fraction, L, lysosomal fraction; P, microsomal fraction; S, soluble fraction.

Figure



that this enzyme appears to be located in the mitochondrial matrix in rat liver.

Figure 9 shows the sub-cellular distribution of the marker enzymes and DNA along with ODC, SDC and OT in liver from GH-treated rats. Ornithine decarboxylase and putrescine dependent SDC appeared to be enriched only in the HSS fraction while ornithine transaminase was enriched only in the mitochondrial fraction. These results are similar to those observed in livers from control rats. Thus, it would appear that growth hormone did not cause an altered distribution of ODC, SDC or OT. GH did, however, markedly increase the specific activity of soluble ODC relative to controls. In 90 g control male rats, the hepatic ODC specific activity was approximately 0.29 nmoles/mg HSS protein/60 min. Four hours after GH injection, the hepatic ODC specific activity was 1.35 nmoles/mg HSS protein/60 min.

Tables 4 and 5 list mean specific activities for all the marker enzymes used in this study, from control and GH treated rats. Four hours after GH injection, the specific activity of these marker enzymes remained unchanged; OT and SDC also showed no significant changes in specific activity after GH treatment. Since both OT (73) and SDC (72) have the same cofactor as ODC (pyridoxal phosphate) and since OT requires the same substrate as ODC, it would appear that the increase in the specific activity of ODC is a relatively specific effect of GH.



Figure 9

Typical distribution pattern of ODC, SDC, OT and marker enzymes, following sub-cellular fractionation of liver from growth hormone treated rat (rat weight: 88g). ordinate: mean relative specific activity of fractions (percentage of total activity/percentage of total protein); abscissa: relative protein content of fractions (cumulatively from left to right). N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, soluble fraction.

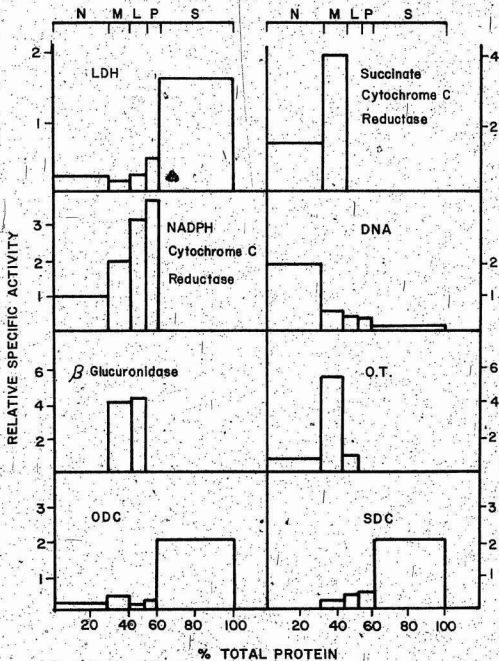


TABLE 4

SPECIFIC ACTIVITIES OF MARKERS FOR SUB-CELLULAR  
FRACTIONS OF LIVERS FROM CONTROL RATS

Fraction	DNA <sup>a</sup>	Succinate Cytochrome C Reductase <sup>b</sup>	Marker (Mean $\pm$ SD, n=5)	$\beta$ -glucuronidase <sup>c</sup>	NADPH Cytochrome C Reductase <sup>b</sup>	Lactate Dehydrogenase <sup>b</sup>
Homogenate	4.68 $\pm$ .48	13.0 $\pm$ 2.6	24.5 $\pm$ 8.1	15.9 $\pm$ 5.3	1020 $\pm$ 110	
Nuclei	19.6 $\pm$ 4.8	16.6 $\pm$ 7.0	16.5 $\pm$ 8.5	9.3 $\pm$ 3.0	140 $\pm$ 40	
Mitochondria	2.18 $\pm$ 0.54	33.0 $\pm$ 8.5	43.9 $\pm$ 1.2	14.3 $\pm$ 1.6	70 $\pm$ 20	
Lysosomes	1.7 $\pm$ 1.0	-	67.5 $\pm$ 5.9	26.4 $\pm$ 8.3	120 $\pm$ 30	
Microsomes	2.93 $\pm$ 0.53	-	14.9 $\pm$ 4.2	43.3 $\pm$ 10.5	360 $\pm$ 100	
Cytosol	1.8 $\pm$ 0.60	-	14.6 $\pm$ 4.9	-	2500 $\pm$ 400	

<sup>a</sup> expressed as  $\mu$ g/mg protein

<sup>b</sup> expressed as nmol/min/mg protein

<sup>c</sup> expressed as nmol/15 min/mg protein

TABLE 5

SPECIFIC ACTIVITIES OF MARKERS FOR SUB-CELLULAR  
FRACTIONS OF LIVERS FROM GROWTH HORMONE TREATED RATS

Fraction	DNA <sup>a</sup>	Succinate Cytochrome C Reductase <sup>b</sup>	Marker (Mean $\pm$ SD, n=4) $\beta$ -glucuronidase <sup>c</sup>	NRAMP Cytochrome C Reductase <sup>b</sup>	Lactate Dehydrogenase <sup>b</sup>
Homogenate	4.57 $\pm$ 0.76	14.0 $\pm$ 4.6	24.8 $\pm$ 2.97	11.1 $\pm$ 2.5	1020 $\pm$ 200
Nuclei	16.0 $\pm$ 2.92	9.5 $\pm$ 4.1	10.6 $\pm$ 1.47	8.18 $\pm$ 2.38	180 $\pm$ 60
Mitochondria	2.61 $\pm$ 0.93	36 $\pm$ 10.4	53.0 $\pm$ 7.07	14.7 $\pm$ 2.9	94 $\pm$ 30
Lysosomes	2.60 $\pm$ 0.38	-	92.1 $\pm$ 19.2	21.7 $\pm$ 3.8	73 $\pm$ 20
Microsomes	2.66 $\pm$ 0.39	-	20.6 $\pm$ 6.2	36.3 $\pm$ 7.5	315 $\pm$ 160
Cytosol	2.09 $\pm$ 1.02	-	14.6 $\pm$ 5.7	-	2000 $\pm$ 500

<sup>a</sup> expressed as  $\mu$ g/mg protein

<sup>b</sup> expressed as nmol/min/mg protein

<sup>c</sup> expressed as nmol/15 min/mg protein

### C. Characteristics of the Enzymes

#### 1) Ornithine Decarboxylase

##### i) Protein concentration

The data presented in Figure 10, demonstrates that assays of hepatic ODC from control and GH-treated rats were linear up to at least 10 mg of HSS protein per 2 ml. of assay medium. Therefore, the amount of HSS protein added to ODC assay mixtures was always kept within 3 to 8 mg of protein, which is well within the linear range of this assay.

##### ii) Time

ODC activity in the HSS was demonstrated to a linear function of time for at least 90 minutes (Figure 11). All experiments in these studies were thus incubated for 60 minutes. As already stated in the introduction, the apparent  $t_{1/2}$  of ODC is approximately 11 minutes, yet we observed linearity up to 90 minutes. It appeared that under our assay conditions the enzyme remained more stable than it is reported to be in vivo. Possibly ODC was protected from degradation by the added substrate or cofactor.

##### iii) Ornithine concentration

Figure 12 shows the effect of increasing ornithine concentration on the velocity of the hepatic ODC activity from both control and GH-treated rats. The Lineweaver-Burk plots of this same data (Figure 14) yielded two straight lines intercepting the abscissa to give  $K_m$  values of 31 and 33  $\mu M$  for the hepatic enzyme from control and GH-treated rats respectively. Hofstee plots of this same data were also drawn (Figure 14). The slopes of the two straight lines were determined to give  $K_m$  values of 32 and 35  $\mu M$  for ODC from control and GH-treated rats. These observed  $K_m$  values are below the ornithine

Figure 10

ODC activity as a function of protein concentration from liver  
of control (x) and growth hormone treated (●) rats.

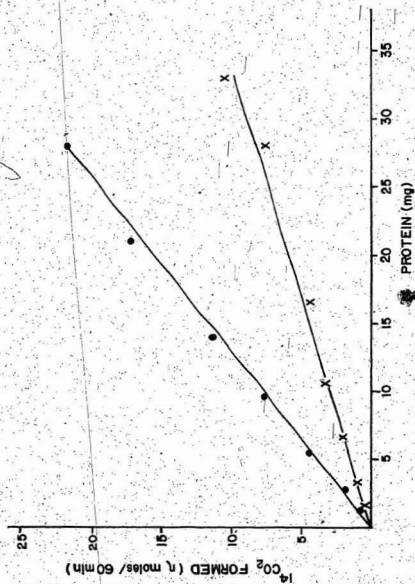


Figure 11

ODC activity in liver MSS, from a control rat, as a function of time.



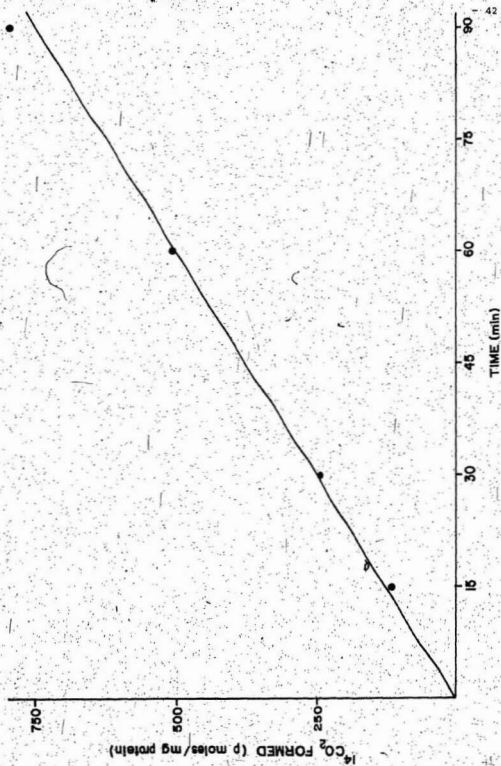


Figure 12

Effect of ornithine concentration on hepatic ODC activity from control (x) and growth hormone (●) rats.

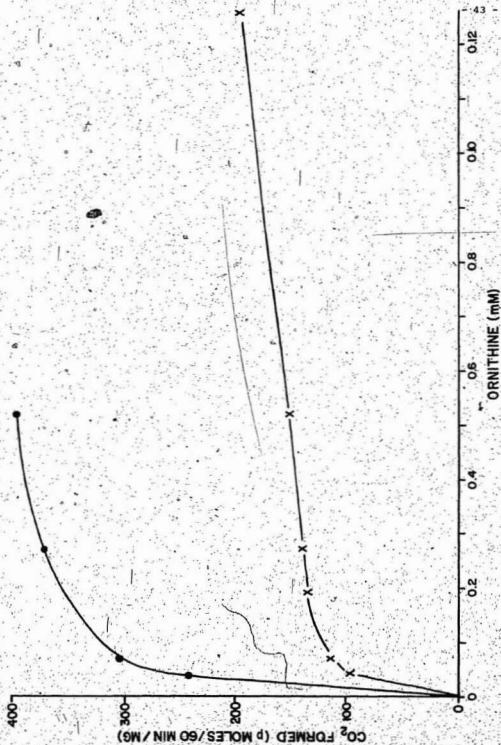


Figure 13

Lineweaver-Burk plot of hepatic ODC activity as a function of  
ornithine concentration from control (●) and growth hormone treated  
(x) rats.  $10^{-4}$  M

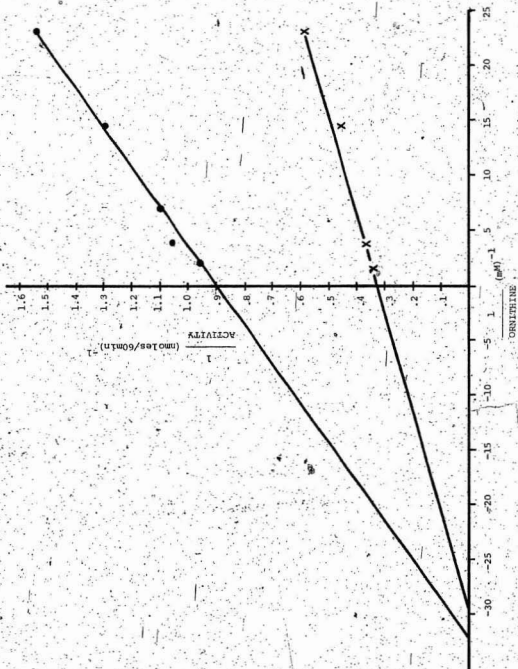
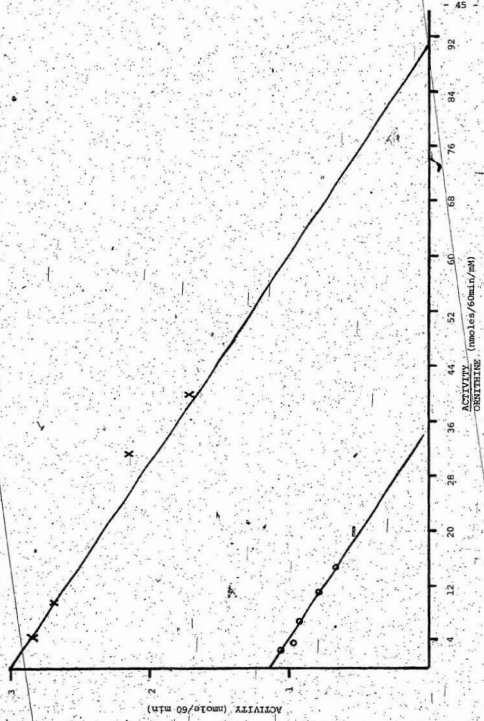




Figure 14

Hofstee plot of hepatic ODC activity as a function of ornithine concentration from control (x) and growth hormone treated (•) rats.



concentration (0.2mM) reported for rat liver by Rajman (85). These results would seem to indicate that the higher specific activity of ODC observed in livers from GH treated rats is not due to any affinity change of ODC for ornithine.

The observed  $K_m$  values reported here are lower than the  $K_m$  (100 $\mu$ M) reported for the rat prostate enzyme (90). This difference in observed  $K_m$  could be due to differences in assay conditions, tissues or breed of rats studied, or to the relative purity of fraction assayed.

#### iv) Pyridoxal phosphate

Supplementation of ODC with added pyridoxal phosphate was studied using dialyzed HSS from liver of control rats. An increase in ODC activity up to about 20 to 30  $\mu$ M pyridoxal phosphate was observed (figure 15). A plateau occurred at higher concentrations of pyridoxal phosphate. It was decided to use 50  $\mu$ M pyridoxal phosphate in all subsequent experiments.

### 2) Putrescine Dependent S-adenosyl Methionine Decarboxylase (SDC)

#### i) Protein concentration

Assays for SDC in liver of normal and of GH treated rats were shown to be linear to 11 mg of HSS protein (figure 16). Assays were always performed within this range.

#### ii) Time

SDC activity in the HSS was linear for at least 90 minutes under the conditions of this assay (figure 17). All assays in these studies were incubated for 60 minutes.

#### iii) Putrescine concentration

The effect of putrescine concentration on the velocity of hepatic



Figure 15

Hepatic ODC activity, from a control rat, as a function of  
pyridoxal phosphate concentration.



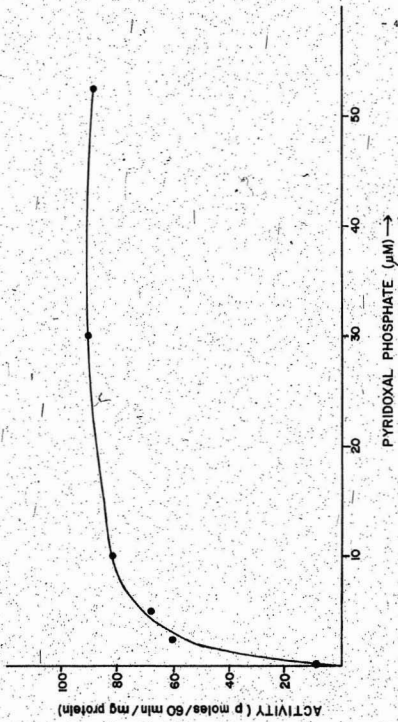


Figure 16

SDC activity as a function of protein concentration from liver  
of control (X) and growth hormone treated (●) rats.

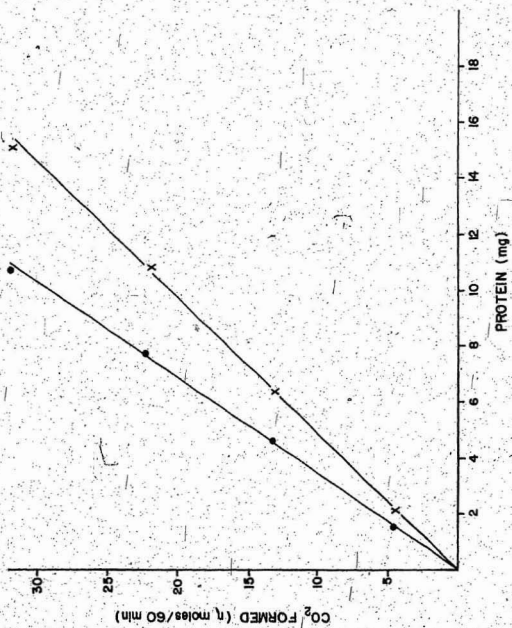
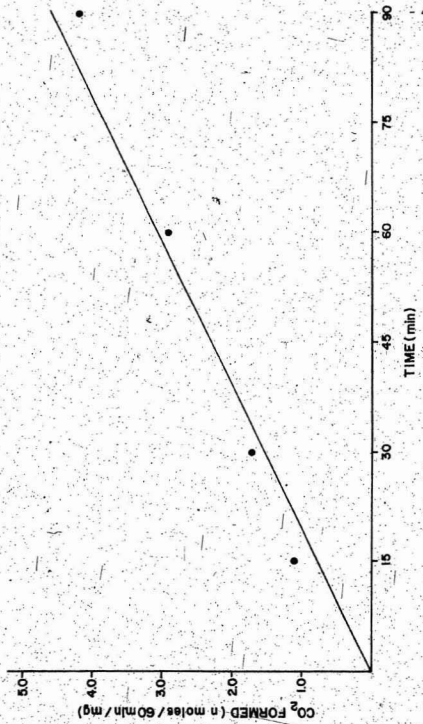


Figure 17

SDC activity in liver HSS as a function of time.



SDC activity was plotted for both control and GH treated rats (Figure 18). The Lineweaver-Burk plots (Figure 19) of these same data yielded the straight lines intercepting the abscissa to give apparent dissociation constants of 0.27 and 0.26 mM for the enzyme in the HSS from control and GH treated rats respectively. Hofstee plots (Figure 20) of this same data were also drawn. The slopes of the two straight lines gave apparent dissociation constants of 0.26 mM for both groups. These results indicate no significant change in the apparent dissociation constant of hepatic SDC for putrescine, 4 hours after GH injection.

Putrescine concentration in liver of control rats was approximately 0.10 mM (around 90g bbdy weight) while a putrescine concentration of 0.23 mM was observed in livers of GH treated rats (of the same weight). These results indicate that SDC would be linearly responsive to putrescine concentration over the range seen from control to GH treated rats.

The SDC assay used in this study measured total enzyme activity at saturating putrescine concentrations. Thus an increase in SDC activity in vivo as a result of increased putrescine concentrations after GH injections would not be measurable by this assay.

Figure 18

Effect of putrescine concentration on the activity of putrescine dependent S-adenosyl methionine decarboxylase from livers of control (X) and growth hormone treated (●) rats.



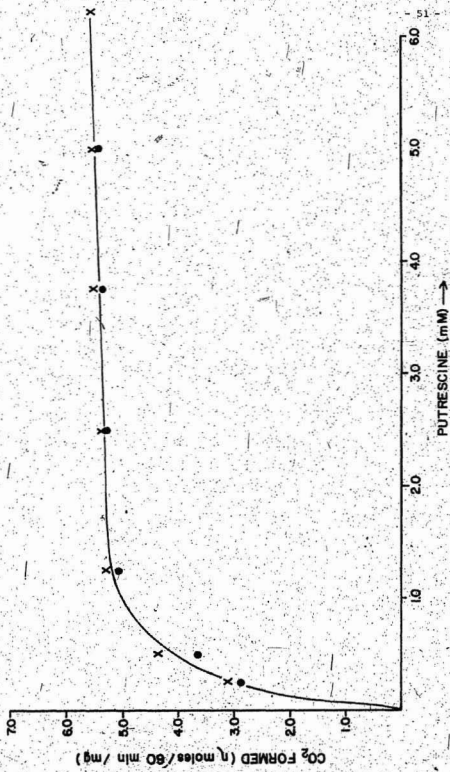


Figure 19

Lineweaver-Burk plot of hepatic SDC activity as a function of putrescine concentration from control ( x ) and growth hormone treated ( ● ) rats. All data points from Figure 18 were not used in this plot.

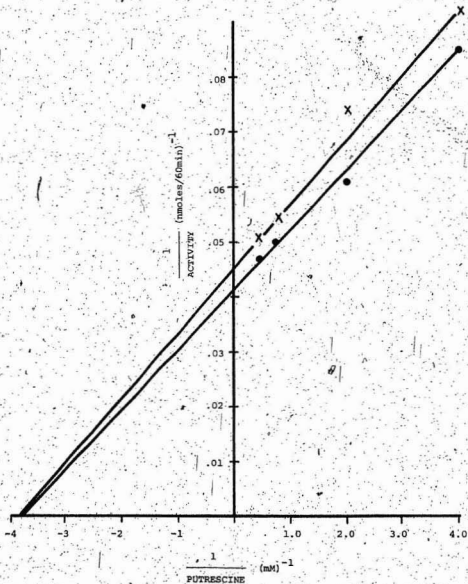
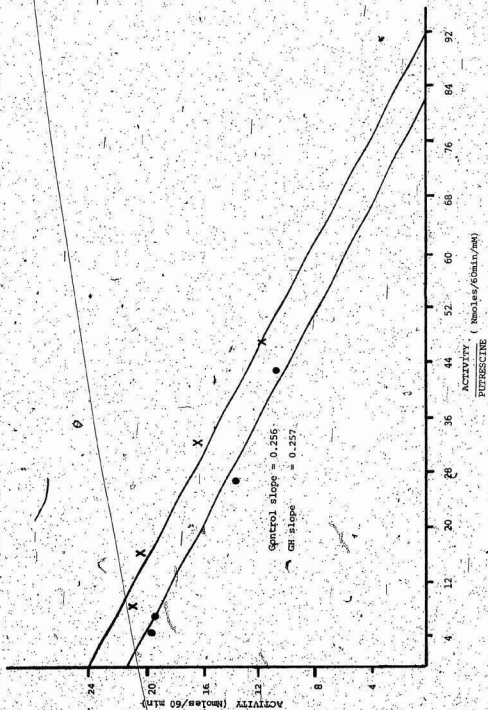


Figure 20

Hofstee plot of hepatic SDC activity as a function of putrescine concentration from control ( x ) and growth hormone treated ( ● ) rats.

All data points from Figure 18 were not used in this plot.



D. Hepatic Ornithine Decarboxylase and Ornithine Transaminase Specific Activity and Polyamine Concentrations in Control and GH Treated Rats of Different Ages

Specific activities of hepatic ODC from HSS and OT from mitochondria of both control and GH treated rats were plotted as functions of rat weight as shown in figures 21 and 23 respectively. The small size and thin skin of the infant rats prohibited injection of GH to very small rats. Homogenate polyamine and HSS putrescine concentrations were also measured in livers of these rats. In separate experiments performed by Mr. Wayne Symonds in this laboratory, spermidine and spermine appeared to bind nonspecifically to membrane fractions at low potassium concentration. For this reason the concentration of these polyamines in HSS was variable and thus is not included here.

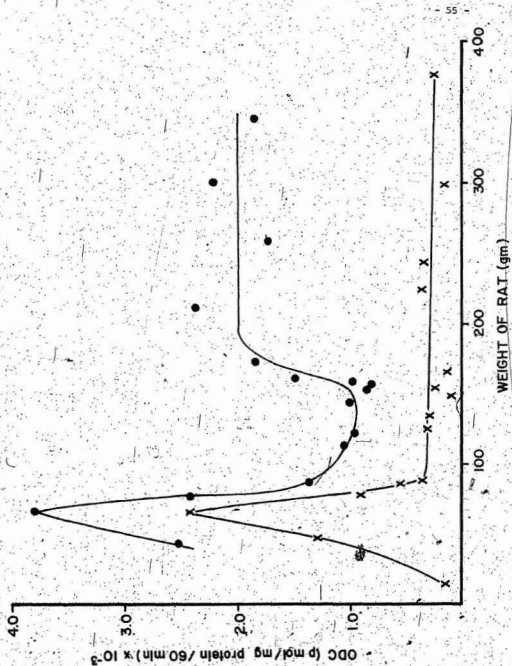
1) Ornithine Decarboxylase

Figure 21 indicates that from 4 days to weaning there was a sharp increase in the specific activity of hepatic ODC from control rats with a peak activity of 2.40 nmoles/mg HSS protein/60 minutes. A rapid decline in specific activity was observed over the next week to a low level of about 0.35 nmoles/mg HSS protein/60 minutes. The observed specific activity then remained relatively constant for as long as it was monitored (approximately 75 days).

Growth hormone treatment resulted in increased total activity of ODC in all of the rats tested from weaning to about 10 weeks of age (Figure 21) when compared to controls of similar ages. However, the increase in specific activity of ODC from GH treated rats displayed a

Figure 21.

Ornithine decarboxylase activity in liver HSS as a function  
of weight of rat: control (x); growth hormone treated (●).  
Each point represents the liver from one rat.



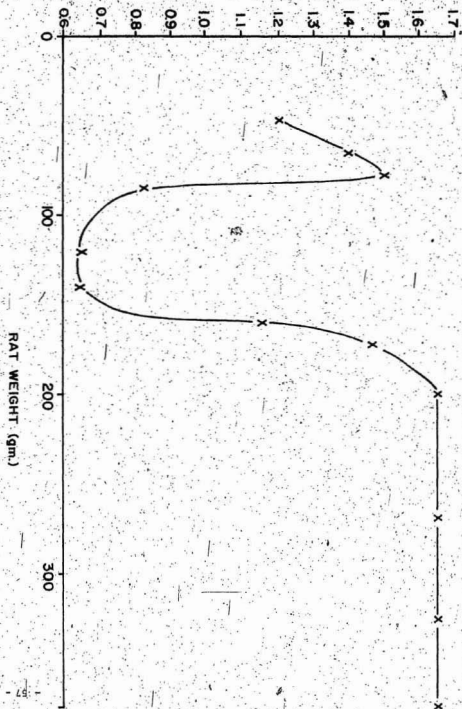


biphasic response (Figure 22). From 20 to 24 days of age (the first few days post-weaning), rats showed a good response to GH as measured by ODC activity. This is just as they are beginning the rapid linear growth phase that lasts well into adulthood. Then from about 25 to 35 days of age there was a much smaller response to the same dose of GH. This was not due to experimental error, since a large number of rats in this age range were studied on different days and always together with younger and/or older rats. By about 36-38 days of age, the response of ODC to GH began to return to levels comparable to those seen in younger rats. This responsiveness was maintained in the oldest rats studied (about 70 days). The diminished responsiveness of the 25-35 day old rats could be due to such factors as decreased sensitivity of liver cells to GH, decreased ability of ODC to respond to stimuli or interference with the effect of GH by another hormone. At this time, it is impossible to predict which factor(s) might be responsible. It is interesting that these rats continue to be responsive to GH, as measured by hepatic ODC activity, at an age when other workers have observed a lack of response to GH in both cartilage (61) and muscle (60). Male rats of this age, however, are capable of responding to injection of GH by an increased growth rate (62). It is also well known that liver continues to respond to elevated GH levels in older animals, such as human, which can not respond with further growth, as evidenced by hepatomegaly (liver enlargement) associated with acromegaly (GH excess) (86). This would be in agreement with the present findings of a continued responsiveness of liver to GH.

Figure. 22

Absolute increase in specific activity of ODC after growth hormone treatment as a function of weight of rat.

ABSOLUTE INCREASE IN SPECIFIC ACTIVITY ( $\mu$ moles/mg/60min)



## 2) Ornithine Transaminase

Ornithine transaminase activity was measured in isolated mitochondria prepared from the livers of rats of different ages (Figure 23). A linear increase in specific activity was observed from approximately 4 days to 4 weeks of age. The activity then remained constant (approximately 180 nmoles  $\Delta^1$  pyrroline-5-carboxylate formed/mg mitochondrial protein/30 minutes) to approximately 6-7 weeks of age, when it began to decline steadily for as long as the rats were studied (about 10 weeks of age).

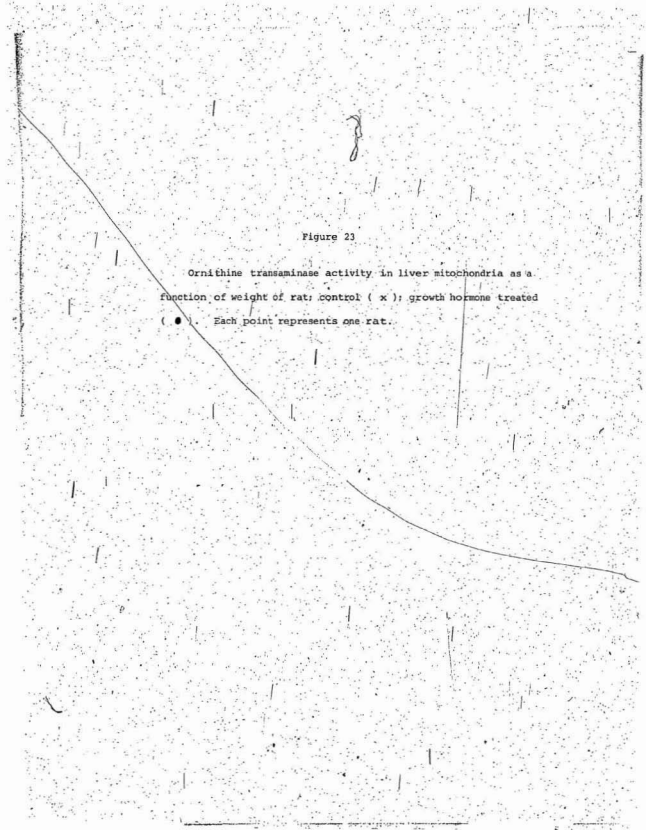
Raiha et al. (87) also observed an increase in OT activity in total liver homogenates from male and female Sprague-Dawley rats 2 to 4 weeks of age. However, they found constant activity from this age into adulthood. They did not observe a decreased activity in the older rats, although they did not state the ages of their "adult" rats. It is possible that they did not follow the rats long enough to observe the decline.

A single injection of GH four hours before sacrifice did not appear to have any effect on OT specific activity, as illustrated in Figure 23. Using repeated injections of GH (3 injections administered at 12 hour intervals), Raiha et al. observed a depression in OT activity per gram liver tissue (12 hours after the last injection). It appears possible that this could be due to a disproportionately larger increase in other liver proteins as a result of GH injections, or possibly it is actually a longer term effect of GH.

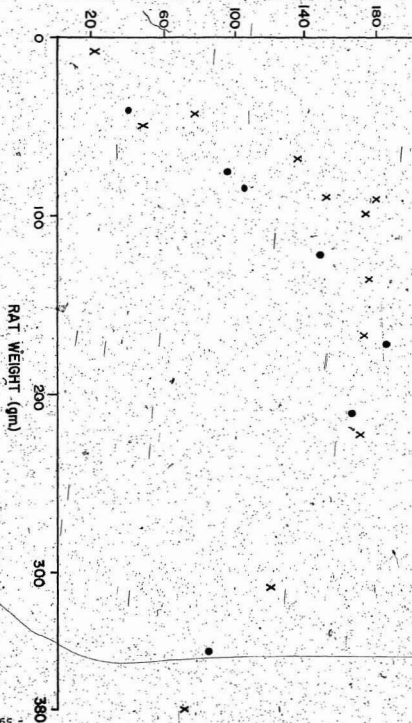
Ornithine transaminase catalyzes the conversion of ornithine to glutamic  $\gamma$  semialdehyde, which is converted to glutamate. This is

Figure 23

Ornithine transaminase activity in liver mitochondria as a function of weight of rat; control ( x ); growth hormone treated ( • ). Each point represents one rat.



ORNITHINE TRANSAMINASE (  $\mu$  moles /mg./ 30 min. )



the first step in the oxidation of ornithine excess to the needs of the liver. As previously stated ornithine also functions as a component in urea biosynthesis (via ornithine carbamyl transferase) and as a precursor for polyamine synthesis (via ODC) in rat liver. Both ornithine carbamyl transferase and ornithine transaminase are much more active than ODC (approximately 100,000 (88) and 1,000 fold respectively). However, both are mitochondrial enzymes and thus would be responsive to mitochondrial ornithine concentrations. As reported in Section B of Results, ODC is a cytoplasmic enzyme, and thus would be responsive to cytoplasmic ornithine concentrations.

### 3) Polyamines

In preliminary experiments, one lobe of rat liver was immediately freeze clamped (92) and ground in liquid nitrogen while five minutes later a second lobe was clamped. Results indicated essentially the same polyamine concentrations in both lobes. Therefore, it was not considered essential to freeze tissue immediately after sacrifice for measurement of polyamine levels. Use of freeze clamping would have required different animals for determinations other than polyamine levels, especially for the small rats (ie. ODC, OT and protein).

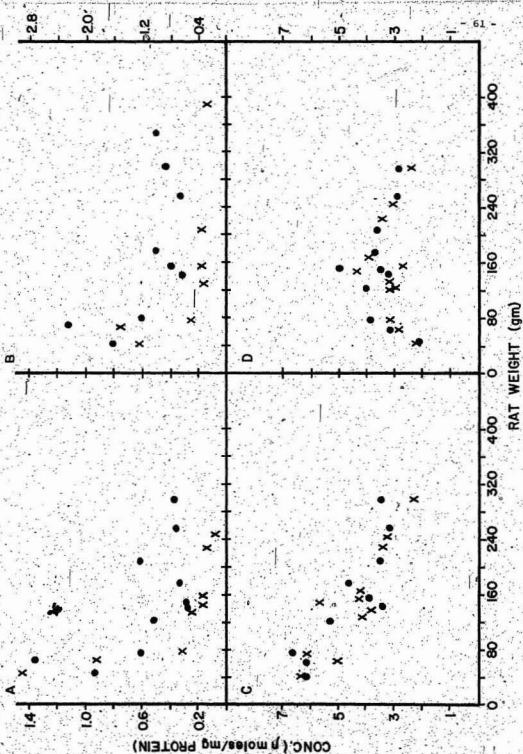
Putrescine concentration (Figure 24) was highest in the youngest rat tested. Forty-six gram control rats (approximately 15 days old) showed a putrescine concentration of 1.45 nmoles/mg homogenate protein. A steady decrease was observed to a low level (0.10 nmoles/mg homogenate protein) by about 6 weeks of age. Liver homogenates from GH treated rats displayed a peak in putrescine

Figure 24

Variation of hepatic polyamine concentrations with weight  
of rat; ( x ) control rats; ( • ) growth hormone treated rats

- a) Putrescine in homogenate
- b) Putrescine in HSS
- c) Spermidine in homogenate
- d) Spermine in homogenate





concentration at 21 days of age (1.50 nmoles/mg homogenate protein), decreasing to a minimal plateau at about 33 days (0.30 nmoles/mg homogenate protein). The hepatic putrescine concentrations from these GH treated rats were about twice as high as those in the livers of control rats. In the HSS fraction putrescine reached a peak concentration at about weaning (1.50 nmoles/mg HSS protein for control rats and 2.20 nmoles/mg HSS protein for GH treated rats) and decreased to a low plateau at about 7 weeks (0.40 nmoles/mg HSS protein for control rats and about 0.80 nmoles/mg HSS protein for GH treated rats). The putrescine concentrations in HSS from GH treated animals were consistently higher than those observed in the HSS fraction from control rats (approximately 1.3 to 2.0 fold increase). This increase in concentration of putrescine in livers of GH treated rats is in agreement with the observed increase in ODC activity 4 hours after injection.

Spermidine concentrations in liver homogenates from control rats appeared to decline steadily from 13 days (6.00 nmoles/mg homogenate protein) to 58 days (2.00 nmoles/mg homogenate protein). A similar decline in spermidine concentrations with age has also been observed by Janne et al. (28). GH injections had no effect on spermidine concentrations 4 hours after injection. Janne (47) observed an increase in 2-<sup>14</sup>C-methionine incorporation into spermidine 6 hours after GH injection, but he did not measure spermidine concentration.

Spermine concentrations in liver homogenate from control rats steadily increased from 13 days to a peak at about 34 days of age (4.50 nmoles/mg homogenate protein), after which a steady decline in concentration was observed to later adulthood. Janne (28) also

found a slight increase in spermine concentration in rat liver from birth to one month of age, but the level then remained unchanged over the next 6 months. GH had no effect on spermine concentrations 4 hours after injection.

## GENERAL DISCUSSION AND CONCLUSION

Ornithine decarboxylase was enriched exclusively in the cytosol fraction of liver from both control and GH treated rats. These results indicate that ODC is not translocated from one cellular compartment to another after GH treatment, as is thought to occur with tyrosine aminotransferase.

It is thought (although unproved) that GH interacts with a specific receptor on the plasma membrane, of the liver cell, and that this interaction is translated into the biochemical response without actual entry of GH into the cell. If this were the case, GH would not be able to interact directly with ODC to change its activity. Thus it is probable that the GH effect on ODC is mediated by a "second messenger", such as cGMP.

We have shown that this increase in ODC activity does not involve a change in sub-cellular distribution or a change in  $K_m$  for ornithine. It is possible that there is a change in  $K_m$  for pyridoxal phosphate or an actual increase in amount of enzyme present. Both of these possibilities are currently being studied in our laboratory.

We have also demonstrated that SDC is exclusively localized in the cytosol fraction of liver from both control and GH treated rats. Since ODC and SDC are cytoplasmic enzymes, it would appear that putrescine and decarboxylated SAM are mainly synthesized in this cellular compartment. The sub-cellular distribution of spermidine and spermine synthetases is not known. If polyamines are indeed affecting nucleic acid synthesis and if spermidine and spermine synthetase were localized at the sites of nucleic acid synthesis:

(nucleus and mitochondria) putrescine and decarboxylated SAM would have to be transported into these compartments. If all these polyamine biosynthetic enzymes were in the cytoplasm then the polyamines would have to be transported to the sites of nucleic acid synthesis, if indeed these amines do have any effect on such processes. There is no information available on the transport of these substrates from one sub-cellular compartment to another, or indeed on the sub-cellular distribution of the polyamines.

A sub-cutaneous injection of GH resulted in an increase in ODC activity, with an approximate 2 fold increase in hepatic putrescine concentration, 4 hours after administration. There appeared to be no change in the total capacity of SDC nor in the concentrations of spermidine and spermine at this time. It is difficult to determine whether GH exerts an in vivo stimulation of SDC since the total in vitro activity was measured using saturating levels of putrescine, a known activator of SDC. However, it should be noted that "blanks" (no putrescine added), for control and GH treated animals did not differ significantly. Even if there were an increase in SDC activity in vivo resulting from GH action, the change in spermidine concentration would probably not be noticeable by four hours since the enzymic activity is not very high and since the  $t_{1/2}$  of spermidine is about 5 days. It would probably take time to build up an increase in concentration that could be detected on the amino acid analyzer.

Weber (88) and Kremzner (89) have independently suggested that OT could control the activity of ODC. If this were the case, an increase in OT activity would result in a decrease in ODC activity by removal of available substrate, and probably an increased

susceptibility of ODC to degradation. Our results indicate that this is not the case. Both enzymes increase in activity during early postnatal development. In older rats, OT levels fall but ODC activity does not increase. We have also shown that ODC and OT are located in different regions of the cell (cytosol and mitochondria, respectively) and thus they would not share the same pool of ornithine as this postulate would assume.

The concentration of ornithine in these two compartments could be regulated by transport of ornithine into, and citrulline out of, mitochondria and/or by arginase activity. Very little is known about the factors which control these processes.

#### Conclusions

- 1) Ornithine decarboxylase activity in liver homogenates can be measured by  $^{14}\text{CO}_2$  formation in the presence of ornithine transaminase by using the transaminase inhibitor aminooxyacetate ( $10^{-5}\text{M}$ ).
- 2) Ornithine decarboxylase and putrescine dependent S-adenosyl methionine decarboxylase appear to be exclusively localized to the cytosol of rat liver.
- 3) Ornithine decarboxylase and putrescine dependent S-adenosyl methionine decarboxylase in growth hormone treated rats appear to be exclusively localized in the cytosol of rat liver.
- 4) Ornithine transaminase appears to be located exclusively in the mitochondrial fraction of liver from both control and growth hormone treated rats.
- 5) The specific activity of ornithine decarboxylase showed a marked increase after growth hormone treatment while putrescine dependent

S-adenosyl methionine decarboxylase and ornithine transaminase showed no changes in specific activity.

- 6) The observed  $K_m$  of hepatic ODC for ornithine was 32  $\mu M$ . GH administration four hours before sacrifice did not affect the affinity of ODC for ornithine.
- 7) The observed dissociation constant of SDC for putrescine as essential activator was 270  $\mu M$ , GH appeared to have no effect.
- 8) Ornithine decarboxylase from rat liver showed an increase in activity from 4 days to weaning. It then declined to a low level 1 week later, which was maintained through adulthood.
- 9) A single injection of growth hormone caused good responses in ODC activity in young rats (4 days to weaning). This response decreased to a low between 25 to 35 days of age but increased again to levels approximately those observed in young rats.
- 10) Ornithine transaminase showed an increase in activity from 4 days to 4 weeks of age. Above 6 to 7 weeks, activity steadily declined. A single injection of growth hormone 4 hours before sacrifice had no effect on activity.
- 11) Putrescine concentrations in hepatic homogenates and HSS fractions of control rats decreased from 15 days to a minimal plateau at about 6 weeks.
- 12) Growth hormone treatment resulted in about a two fold increase in putrescine concentrations from both the homogenate and HSS fractions of all ages tested.
- 13) Hepatic homogenate spermidine concentrations showed a marked decrease from 13 to 58 days. Growth hormone had no effect on spermidine concentrations.

- 14) Hepatic homogenate spermine concentrations increased from 13 to 34 days of age, but declined as the rats aged. Growth hormone showed no effect on spermine concentrations.



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